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Characterization of nucleotide binding sites on the membrane-bound chloroplast ATP synthase (coupling factor CF_1)

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Phosphorylation of ADP and nucleotide exchange by membrane-bound coupling factor CF_1 are very fast reactions in the light, so that a direct comparison of both reactions is difficult. By adding substrate ADP and phosphate to illuminated thylakoids together with the uncoupler FCCP, the phosphorylation time is limited and the amount of ATP formed can be reduced to less than 1 ATP per enzyme. Low concentrations of medium nucleotides during illumination increase the amount of ATP formed during uncoupling presumably by binding to the tight nucleotide binding site (further designated as 'site A') with an affinity of 1 to 7 μM for ADP and ATP. ATP formation itself shows half-saturation at about 30 μM. Loosely bound nucleotides are exchanged upon addition of nucleotides with uncoupler (Schumann, J. (1984) Biochim. Biophys. Acta 766, 334–342). Release depends binding of nucleotides to a second site. The affinity of this site for ADP (in the presence of phosphate) is about 30 μM. It is assumed that phosphorylation and induction of exchange both occur on the same site (site B). During ATP hydrolysis, an ATP molecule is bound to site A, while on another site, ATP is hydrolyzed rapidly. The affinity of ADP for the catalytic site (70 μM) is in the same range as the observed Michaelis constant of ADP during phosphorylation; it is assumed that site B is involved in ATP hydrolysis. Site A exhibits some catalytic activity; it might be that site A is involved in ATP formation in a dual-site mechanism. For ATP hydrolysis, however, direct determination of exchange rates showed that the exchange rate of ATP bound to site A is about 30-times lower than ATP hydrolysis under the same conditions.

Introduction

During illumination of chloroplasts, formation of ATP is catalyzed by the membrane-associated ATP synthase complex [1,2]. The energy stored in the electrochemical potential difference induces reactions of the membrane-associated CF_1 part of the ATPase complex which converts ADP and phosphate into ATP [3,4]. The CF_1 contains binding sites for nucleotides [5–7]; one ADP molecule is tightly bound to the coupling factor complex of deenergized membranes which remains bound even after isolation of the CF_1 [8–10]. Other binding sites were described for the isolated but not for the membrane-bound enzyme [1].

On the isolated CF_1 part, three types of nucleotide binding sites were found [6]; site 1 contains the tightly bound ADP molecule which

Abbreviations: AdN, adenine nucleotide(s); CF_1, chloroplast coupling factor 1; Chl, chlorophyll; P_i, inorganic phosphate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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exchanges slowly for medium ADP, or ATP. Site 2 specifically binds an ATP molecule (together with magnesium) which is not exchanged during catalysis. It is unlikely that site 1 or site 2 are catalytic sites, although site 1 shows some ATP hydrolyzing activity [6,10]. Another binding site (site 3) was found on the isolated CF_1 for reversible binding of adenine nucleotides; this site is presumably the catalytic site of the isolated enzyme [6]. Similar results predicting three tight nucleotide sites were found by Girault et al. [7].

Besides the tight ADP site, no further binding sites for adenine nucleotides were described for the membrane-bound coupling factor under deenergized conditions [11]. The exchange rate of this bound ADP is slow in the dark; upon energization by illumination, by acid-base transitions or by external electric fields [8,12,13], the exchange is accelerated. During illumination of thylakoids in the presence of medium ADP or ATP, an equilibrium between binding and release of nucleotides on the 'tight' site is established; the dissociation constant for ADP or ATP is about 2–8 μM [14–16]. It is unknown whether this site participates in the process of ATP formation or ATP hydrolysis; at least some ATPase activity of this site was described [6,10].

The Michaelis constant for ADP in ATP synthesis is about 10-times higher than the dissociation constant of the tight site [17]. It was therefore assumed that two different types of nucleotide binding site exist on the coupling factor; however, an apparent Michaelis constant may be higher than a dissociation constant of the same site. Indeed, a lower K_m for ADP was found at lower V_{max} rates of phosphorylation [11,18,19]; the extrapolation gave K_m values in the micromolar range.

Since only one nucleotide binding site was found on the coupling factor of illuminated thylakoids (with ADP concentrations up to 10 μM), it was assumed that the tight site might be a different form of the catalytic site [11].

A second type of nucleotide sites was postulated on the activated CF_1 because in the presence of medium nucleotides, the release of tightly or loosely bound ADP from the first site is accelerated [16,20]. ATP hydrolysis occurs even with ATP bound to the tight binding site [21]. During photophosphorylation, binding of phosphate and ADP induces a rapid release of newly synthesized ATP from its binding site [22,23]. On the basis of these experiments, a hypothesis of cooperative binding sites ('energy-linked binding change mechanism'. Refs. 24 and 25) was developed: after binding of ADP and phosphate to one site, an energy-dependent conformational change releases tightly bound ATP from another site, while the first site (with ADP and phosphate bound) is converted into a tight site. On this site, ATP formation occurs without energy input. The release of tightly bound nucleotides was found to be fast enough for the catalytic process of ATP formation or hydrolysis [24].

In this paper, the properties of nucleotide binding sites on the coupling factor were investigated during and after illumination; the data suggest that the tight/loose binding site has at least some catalytic activity. Another type of site on the coupling factor binds nucleotides with lower affinity and is assumed to have catalytic properties. This is clearly demonstrated for light-triggered ATP hydrolysis, where ATP is hydrolyzed on the catalytic site while another site is empty or contains an ATP molecule without affecting the activity of the enzyme.

Materials and Methods

Broken chloroplasts were isolated from spinach leaves as described [9]. Chlorophyll concentrations were determined according to Arnon [26]. Incubation of the chloroplast suspensions was carried out in small glass vessels under stirring with a magnetic stirrer. The assay medium contained Tricine buffer (25 mM, pH 8), NaCl (50 mM), $MgCl_2$ (1 mM), phenazine methosulfate (50 μM), and thylakoids (chlorophyll concentration, 50–200 μg/ml). The samples were illuminated from the top (200 W/m^2, white light) for 30–60 s.

For experiments with magnesium-free thylakoids, chloroplasts were isolated without $MgCl_2$ but with EDTA (1 mM) in the isolation media. The assay medium contained neither $MgCl_2$ nor EDTA.

For the determination of hydrolysis rates, preillumination was carried out in the presence of dithioerythritol (5 mM).

Binding of [^{14}C]nucleotides. For measurements of [^{14}C]AdN binding to the nucleotide-depleted membranes, samples were preilluminated in the absence of nucleotides, and [8-^{14}C]ADP or -ATP (2.1 GBq/mmol; Amersham-Buchler) was added together with an FCCP solution in the light. FCCP was added as an ethanolic solution; the final concentration of ethanol was 5% or less. Binding of labeled nucleotides was terminated by the addition of excess unlabeled ADP (5–10 mM). The amount of tightly bound nucelotides was determined after chloroplast washing as described in Ref. 9.

For measurements of [^{14}C]nucleotide levels in the light or after light-to-dark transitions, preillumination was carried out in the presence of labeled nucleotides. To prevent extensive hydrolysis of labeled ATP in the light, [^{14}C]ATP was added 5 s before switching off the light.

Exchange of loosely bound nucleotides during uncoupling. 'Loosely bound nucleotides' are those nucleotides which are bound to the nucleotide binding site during illumination [14,16]; they are converted to tightly bound nucleotides if an uncoupler is added without unlabeled nucleotides. If a high concentration of unlabeled ADP is added together with an uncoupler in the light, these nucleotides are released into the medium. Under these conditions, only 'tightly bound nucleotides' remain bound to the membranes. Determination of the amount of tightly and loosely bound nucleotides is performed by the addition of FCCP to thylakoids which are illuminated in the presence of [^{14}C]nucleotides. Binding of labeled medium nucleotides to the deenergized membranes shortly after the addition of the uncoupler is prevented by the addition of unlabeled ADP a few seconds after the first addition. The amount of exchanged nucleotides is the difference between this value and the value obtained under the given experimental conditions (e.g., addition of FCCP together with a suboptimal concentration of unlabeled ADP).

Formation of [^{32}P]ATP during uncoupling. To determine the amount of ATP formed during the addition of substrates and the uncoupler to preilluminated chloroplasts, ADP (1 mM) and [^{32}P]phosphate (0.5–1 mM; about 1 GBq/μmol, Amersham-Buchler) was added together with FCCP in the light. 5 s later, perchloric acid was added (final concentration, 0.3 M), and the amount of labeled ATP was determined after precipitation of free phosphate [27].

Determination of ATP hydrolysis rates. For the determination of hydrolysis rates, [γ-^{32}P]ATP was added to samples preilluminated in the presence of dithioerythritol (5 mM). The reaction was stopped with perchloric acid, and released [^{32}P]phosphate was extracted with isobutanol/toluene [28].

Radioactivity was measured in liquid scintillation cocktail (Unisolve 1, Koch-Light Lab., or Ready-Solv, Beckman) or in water (Cerenkov counting of ^{32}P).

Results

Nucleotide binding and phosphorylation during uncoupling

It was described in a preceding paper that phosphate increases binding of ADP to the energized thylakoids when added together with FCCP in the light (Ref. 29 and Table I). The effect depends on phosphorylation, but it remained unclear whether the additional amount of bound ATP was synthesized on the same site or originated from newly synthesized medium ATP.

TABLE I

EFFECT OF MAGNESIUM AND PHOSPHATE ON TIGHT BINDING OF [^{14}C]ADP AND [^{14}C]ATP TO ILLUMINATED THYLAKOIDS

Chloroplasts were isolated without $MgCl_2$ according to Materials and Methods; $MgCl_2$ was omitted from the illumination medium. Chlorophyll concentration during illumination was 120 μg/ml. After 30 s in the light, radioactive ADP or ATP (final concentration, 5 μM) was added together with FCCP (5 μM) and, when indicated, phosphate (1 mM) and/or $MgCl_2$ (1 mM). 2 s later, unlabeled ADP was added to a final concentration of 5 mM, and the amount of bound [^{14}C]nucleotides was determined.

Additions:	Bound nucleotides (nmol per mg Chl)	
	[^{14}C]ADP	[^{14}C]ATP
No addition	0.166	0.078
Mg^{2+}	0.149	0.085
P_i	0.146	0.032
$Mg^{2+} + P_i$	0.242	0.050

If [^{14}C]ATP is added instead of [^{14}C]ADP, less nucleotides are bound during the initial phase (Table I). Phosphate decreases the amount of bound nucleotides even further. It is therefore unlikely that on addition of [^{14}C]ADP plus phosphate and FCCP, ATP is synthesized on another site and is transferred through the medium to the tight site. Tightly bound ATP after addition of ADP therefore indicates catalytic activity of the tight binding site.

To determine the total amount of ATP synthesized during the addition of ADP, phosphate and FCCP, ATP formation during uncoupling was investigated using radioactive phosphate. Preilluminated thylakoids were supplied with substrate ADP and [^{32}P]phosphate together with FCCP in the light, and perchloric acid was added 5 s later. In this type of experiment, the amount of formed ATP can be limited to less than one ATP per CF_1 while the kinetic constants of phosphorylation are unchanged. For example, variation of the ADP (or phosphate) concentration in the quench solution (at a fixed FCCP concentration) gives normal saturation curves; identical Michaelis constants were obtained at different concentrations of the uncoupler (not shown). The apparent Michaelis constant for ADP is 30–50 μM (see Fig. 3), while the respective constant for phosphate is 250–400 μM. These values are comparable to those obtained for steady-state phosphorylation during continuous illumination [30].

Even if 25 μM FCCP were added together with 1 mM ADP and 1 mM phosphate, detectable amounts of ATP were formed. Under these conditions, 4.5 nmol ATP were formed per mg chlorophyll, i.e., about 4 ATP per CF_1 [31]. If a low concentration of ADP ('medium ADP'; 10 μM) was present in the light, and a high concentration of ADP ('substrate ADP') was added with the uncoupler and phosphate after 30 s, the amount of ATP formed was increased (9.3 nmol/mg Chl). The low concentration of medium ADP does not serve as substrate ADP for ATP formation, since medium ATP is able to replace medium ADP (see Fig. 1).

This effect of medium nucleotides is saturated at low concentrations (Fig. 1); the half-maximal concentration being 5 μM ADP or ATP. The concentration of nucleotides needed for the

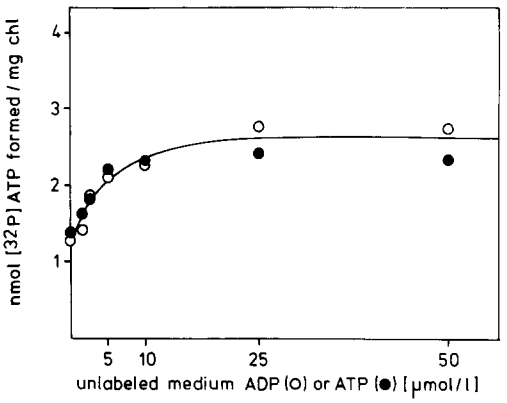

Fig. 1. Effect of low concentrations of medium nucleotides on the formation of ATP during uncoupling. Chloroplasts (100 μg/ml) were illuminated in the presence of varying concentrations of unlabeled ADP or ATP. After 30 s, FCCP (50 μM) was added together with a high amount of ADP (1 mM) and [^{32}P]phosphate (1 mM). Perchloric acid was added 2 s later, and the amount of [^{32}P]ATP was determined.

enhancement of ATP formation is comparable with the dissociation constant of the tight nucleotide binding site during illumination [14,16]. Obviously, the observed effect depends on binding of ADP or ATP to the (tight) nucleotide site of the energized form of the coupling factor (presumably by increasing the proton gradient in the presence of low concentrations of nucleotides [32]).

Nucleotide release and ATP formation

As described in a previous paper [16], a certain amount of loosely bound nucleotides is exchanged upon addition of FCCP and excess nucleotides. In this paper, the properties of this exchange reaction were compared with the formation of ATP under the same conditions.

In an experiment shown in Fig. 2, the effect of varying FCCP concentrations on the formation of ATP as well as the release of nucleotides was studied. For the determination of exchangeable nucleotides, thylakoids were preillimunated in the presence of [^{14}C]ADP; after 30 s, unlabeled ADP and phosphate were added together with varying concentrations of the uncoupler.

In a parallel series, the extent of ATP formation was determined; unlabeled ADP was employed during illumination, while substrate ADP and radioactive phosphate were added together with the uncoupler.

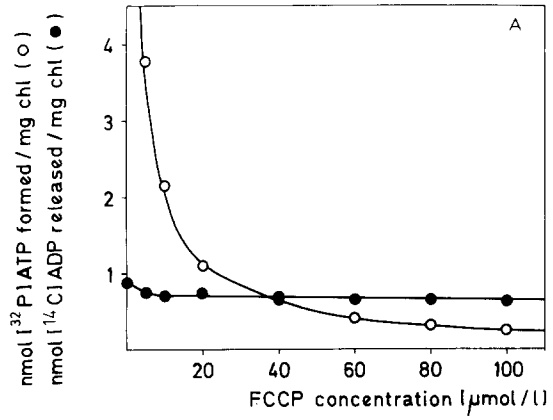

Fig. 2. Formation of ATP and release of loosely bound nucleotides during addition of FCCP. Chloroplasts (127 μg/ml) were illuminated for 30 s in the presence of ADP (5 μM). For the determination of [^{32}P]ATP formation (closed cycles), a solution of ADP (1 mM), [^{32}P]P_i (1 mM), and various concentrations of FCCP was added in the light; the reaction was stopped with perchloric acid 2 s later. For the determination of [^{14}C]ADP binding, illumination was carried out in the presence of [^{14}C]ADP instead of unlabeled ADP. After 30 s, a solution of ADP (1 mM), unlabeled P_i (1 mM), and FCCP was added, and the amount of bound [^{14}C]ADP was determined. The amount of exchanged nucleotides was calculated as the difference to the total amount of loosely bound nucleotides (see Materials and Methods).

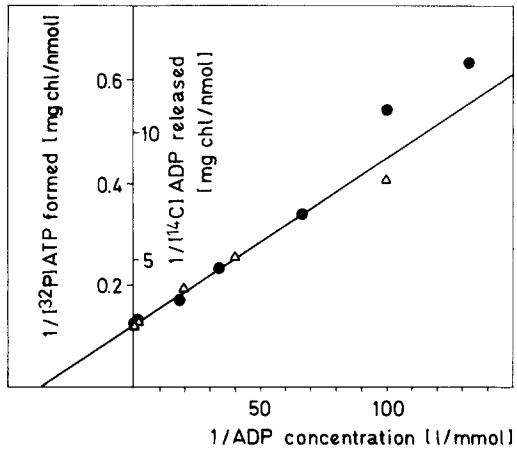

Fig. 3. ATP formation and ADP release during uncoupling in the light as a function of the ADP concentration in the quench. Conditions were as in Fig. 2, except that the ADP concentration in the quench solution was varied, and the FCCP concentration was kept constant (10 μM). Chlorophyll concentration was 136 μg/ml. To prevent slow binding of [^{14}C]ADP after the addition of FCCP, ADP and phosphate, a high concentration of ADP (5 mM) was added to the [^{14}C] samples. ●, ATP formation; △, ADP release.

In Fig. 2, the amount of nucleotides exchanged during the quench and the amount of ATP formed under the same conditions is shown. Exchange of loosely bound nucleotides is almost unaffected by the concentration of FCCP used in this experiment (see also Ref. 16). As determined from the reciprocal plot, 0.6 nmol [^{14}C]ADP are still exchangeable at infinite FCCP concentration. In contrast, the formation of ATP extrapolates to zero at infinite uncoupler concentration. Variation of the uncoupler concentration gives therefore different, low amounts of synthesized ATP at nearly constant values of exchanged nucleotides.

In the next experiment, the concentration of FCCP was kept constant, while the concentration of ADP (added together with phosphate and the uncoupler) was varied. The amount of [^{14}C]ADP exchanged by unlabeled ADP as well as the amount of [^{32}P]ATP formed during the quench were plotted in a reciprocal plot (Fig. 3).

Both processes follow the same saturation curve, with half-maximal effects at about 30 μM ADP. This result shows that the enzyme containing a (loosely) bound adenine nucleotide (binding constant about 5 μM) has a second binding site with lower affinity fo ADP (30 μM). Binding of ADP and phosphate to this site leads to the ejection of the nucleotide from the first site as well as to phosphorylation of medium ADP.

ATP release and light-triggered ATP hydrolysis

Early experiments on the effect of ADP binding to the light-triggered ATPase showed that the enzyme form containing a nucleotide-free binding site is active in ATP hydrolysis, while the enzyme species containing tightly bound ADP is inactive [33]; this result was also found by other groups [34,35]. With tightly bound ATP presumably at the same site, however, no inhibition of the light-triggered ATPase was observed [21], indicating that under those conditions even with an occupied binding site, the enzyme is still capable of ATP hydrolysis.

In a preceding paper [33], the inhibitory effect of ADP on the rate of ATP hydrolysis was investigated. ADP was added either before, together with or after the addition of substrate ATP. The

extent and type of inhibition as well as the K_i values were found to depend on the time of ADP addition.

In the experiment shown in Fig. 4, thylakoids were illuminated either in the presence or absence of a low concentration of ATP. After light-to-dark transition, [γ-^{32}P]ATP was added together with varying concentrations of ADP, and the rate of ATP hydrolysis was determined. If ATP was present in the light, competitive inhibition with a K_i (ADP) of about 60 μM was apparent; without ATP in the light, the K_i(ADP) was 5 μM. Under the latter conditions, the simultaneous addition of ADP and ATP to the nucleotide-free membranes may lead to irreversible binding of ADP to some of the enzyme molecules, thus causing inactivation of those ATPases (under the experimental conditions, irreversible inactivation of some ATPases gives a quasicompetitive type of inhibition because added ADP competes with added ATP for the tight binding site; see below). ATP – like ADP – is tightly bound to the coupling factor at a light-to-dark transition [10,14–16]. ATP binding, however, does not inactivate the ATPase. Assuming that ATP binds to the same site as ADP (see Ref. 21), it is obvious that an enzyme containing bound ATP is protected from inactivation by ADP.

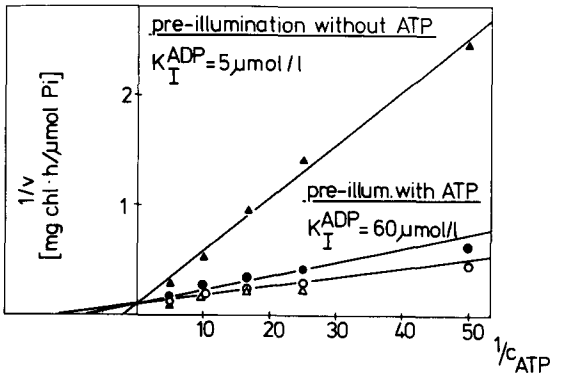

Fig. 4. Inhibition of light-triggered ATPase by ADP. Chloroplasts (46 µg/ml) were illuminated for 1 min; the medium contained in addition dithioerythritol (5 mM). When indicated, unlabeled ATP (10 µM) was added 5 s before switching off the light. After 10 s in the dark, [^{32}P]ATP was added with or without ADP (25 µM). ATP hydrolysis was stopped after 10–30 s with perchloric acid. ○, illumination with ATP; hydrolysis without ADP; ●, illumination with ATP; hydrolysis with ADP; △, illumination without ATP; hydrolysis without ADP; ▲, illumination without ATP; hydrolysis with ADP.

The K_i value for ADP found after loading ATP onto the tight binding site (60 μM, see Fig. 4) is probably the actual competitive inhibitor constant indicating the affinity of the catalytic (ATP hydrolyzing) site for ADP.

In the next part, the fate of bound ATP during ATP hydrolysis was investigated. In the experiment shown in Fig. 5, thylakoids were illuminated in the presence of 5 μM [^{14}C]ATP. After switching off the light, either unlabeled ADP together with FCCP (open symbols), or unlabeled ATP (closed symbols) was added at different times. The amount of bound radioactive nucleotides was determined after several minutes in the dark.

With ADP and FCCP in the quench, normal biphasic binding kinetics were obtained [14,16]. If ATP was added without FCCP (i.e., conditions under which ATP hydrolysis occurs), a much lower level of bound nucleotides was found. Therefore, ATP is released during hydrolysis of medium ATP but remains bound after deenergization (i.e., addition of ADP plus uncoupler).

To measure the exchange rate of bound [^{14}C]ATP induced by light-triggered ATP hydrolysis, thylakoids were illuminated in the presence of radioactive ATP, and a limited amount of unlabeled ATP was added 10 s after illumination.

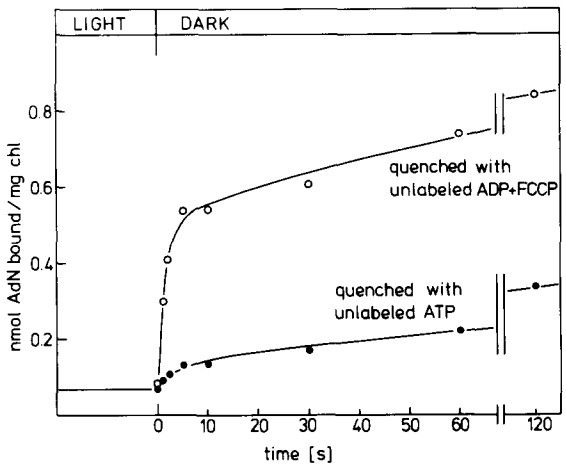

Fig. 5. Time-course of [^{14}C]ATP binding during light-to-dark transitions. Illumination (+dithioerythritol) as in Fig. 4; chlorophyll concentration was 147 µg/ml. [^{14}C]labeled ATP (5 µM) was added 5 s before switching off the light. Binding of ATP was quenched by the addition of either unlabeled ADP and FCCP (5 mM and 10 µM, respectively), or unlabeled ATP (5 mM). Thylakoids were washed after 30 min in the dark.

At various times the reaction was terminated by addition of a high concentration of ADP, FCCP and EDTA which stops the ATPase reaction almost immediately. In a parallel series, [^{32}P]ATP was added, and the time-course of ATP hydrolysis was followed by the release of [^{32}P]phosphate.

The level of bound [^{14}C]AdN decreased after the addition of ATP. From the slope of the curve, an initial exchange rate of 0.45 μmol AdN/mg Chl/h was calculated. Using the ATP content at zero and 0.5 s after ATP addition (0.76 and 0.62 nmol/mg Chl), a release rate of 1 μmol/mg Chl per h is apparent. The rate of ATP hydrolysis was 16 μmol ATP/mg Chl per h within the first minute after the addition of ATP; no lag phase was observed. ATP hydrolysis appears to be about 15- to 30-times faster than the release of bound nucleotides (Fig. 6).

If the light trigger is done in the absence of medium ATP, the tight binding site is depleted. When ATP is added in the dark, ATP binding as well as hydrolysis occurs simultaneously. Fig. 7 demonstrates that under these conditions, the rate of ATP hydrolysis is relatively fast (2 μmol P_i/mg Chl per h; upper part) while ATP binding is slow (0.06 μmol AdN bound/mg Chl per h; lower part). In a parallel series, FCCP was added 5 s after the light trigger; ATP was added 5 s later. While ATP binding to the deenergized coupling factor is not affected by the uncoupler, the rate of ATP hydrolysis is slower than in the control, and the reaction stops before ATP is completely hydrolyzed (see Refs. 36, 37).

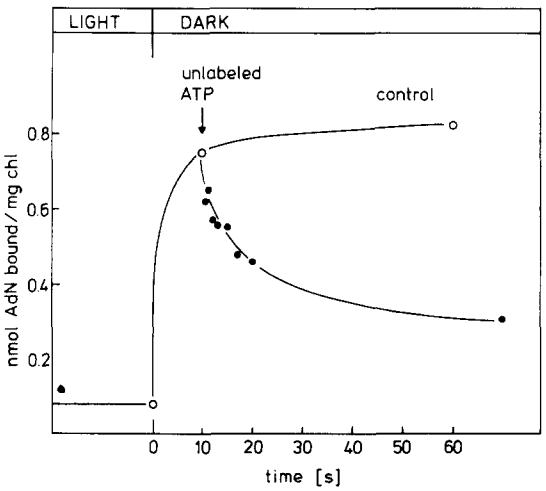

Fig. 6. Time-course of [^{14}C]ATP binding and release. Conditions as in Fig. 5; chlorophyll concentration 129 μg/ml. After 10 s in the dark, unlabeled ATP (200 μM) was added to start ATP hydrolysis. The reaction was stopped by a solution of ADP (10 mM), FCCP (10 μM) and EDTA (5 mM). For the control (open cycles), no ATP was added after 10 s in the dark.

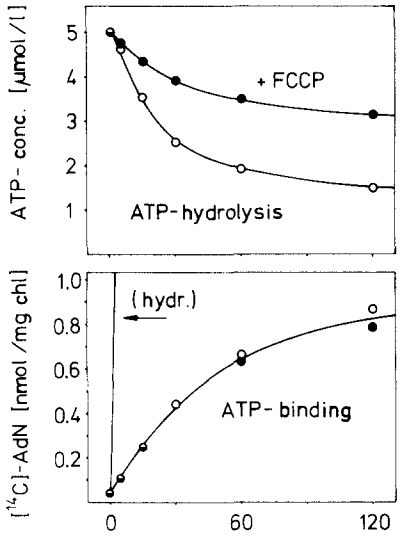

Fig. 7. Time-course of ATP binding to preilluminated thylakoids and of ATP hydrolysis. Thylakoids (150 μg/ml) were illuminated for 1 min in a medium containing dithiothreitol; either 5 μM [^{14}C]ATP (lower part) or 5 μM [^{32}P]ATP (upper part) was added 10 s after illumination. Binding was terminated by the addition of unlabeled ADP and FCCP; hydrolysis was terminated with perchloric acid. Closed symbols: FCCP (final concentration 5 μM) was added 5 s after the light trigger and 5 s before ATP addition.

From these results it is assumed that the appearance of bound ATP is not required for ATP hydrolysis; i.e., hydrolysis is not affected by the presence or absence of ATP on the tight site.

Discussion

The data described in this paper – in accordance with the literature – strongly support a model of at least two different types of nucleotide binding site on the chloroplast coupling factor. One type (further designated as 'site A') binds adenine nucleotides with high affinity and has some catalytic activity. Since the release of bound nucleotides is accelerated in the presence of medium nucleotides, and light-triggered ATP hydrolysis is possible either without or with ATP

bound to site A, at least one additional type of site ('site B') is assumed on the coupling factor of illuminated or preilluminated thylakoids; this site might be the main catalytic site in ATP formation and hydrolysis.

On deenergized thylakoids, site A contains an ADP molecule. Hardly any release or exchange of this ADP occurs in the dark; upon illumination in the absence of medium nucleotides, this bound ADP is released rapidly, thus leading to a nucleotide-free enzyme form [9]. This site binds ADP or ATP during or after energization of thylakoids; the rate of nucleotide binding is higher in the light than in the dark [16]. Re-release of bound AdN makes it impossible to determine absolute binding rates to the energized conformation of the CF_1, but relative numbers were obtained by the addition of radioactive nucleotides and an uncoupler to preilluminated thylakoids (Ref. 16 and this paper).

Phosphate increases the amount of bound nucleotides when added together with ADP and the uncoupler; ATP is formed and remains bound under these conditions [29]. Since binding of medium ATP is slow, and is further decreased in the presence of phosphate (Table I), it is assumed that this bound ATP was formed on the tight site itself, thus showing catalytic activity of this site. ATPase activity of presumably the same site was shown earlier by Magnusson and McCarty [10].

Binding of ADP to the nucleotide-depleted site A is nearly irreversible in the dark [9]. [^{14}C]ATP behaves like [^{14}C]ADP unless substrate ATP is added, allowing ATP hydrolysis: under these conditions, bound [^{14}C]ATP is released (Figs. 5 and 6). Since ATP release is slower than ATP hydrolysis, it seems obvious that cleavage of ATP occurs on a second site without direct participation of the nucleotide-containing site A. A possible explanation for the slow release (Fig. 6) is the proton gradient formed during light-triggered ATP hydrolysis, which even drives phosphorylation of medium ADP [38].

Under energized conditions, reversible binding of nucleotides to the tight binding site (site A) is obtained; the affinity of this site for ADP is about 3–7 μM [14,16], and about 1–7 μM for ATP [15]. A portion of the nucleotides are loosely bound, but even in the light, some tightly bound nucleotides are present [14,16]. Assuming that the enzyme form containing tightly bound ADP is inactive in photophosphorylation [11], the enzyme form containing loosely bound ADP is responsible for ATP formation in the light.

Upon deenergization by switching off the light or by adding an uncoupler, all enzymes containing loosely bound nucleotides change to a conformation with tightly bound nucleotides. If a high amount of ADP or ATP is added together with the uncoupler, all loosely bound nucleotides are released, indicating again that at least one other binding site for nucleotides is present on the coupling factor of energized thylakoids. This binding site ('site B') has a lower affinity for ADP or ATP than site A (30–60 μM versus 1–7 μM; see Figs. 3 and 4), and it catalyzes light-triggered ATP hydrolysis even with ATP bound to site A (Figs. 6 and 7).

It is reasonable that rapid ATP formation in the light is catalyzed by site B, because the apparent Michaelis constant for ADP during ATP synthesis is in the same range as the K_i(ADP) in ATP hydrolysis. The same concentration of ADP is needed for the half maximal ejection of ADP (or ATP) from site A (Fig. 3), indicating that the affinity of site B is lower than that of site A. However, a direct interaction between site A and site B is evident for the energized enzyme.

Cooperative effects between binding sites of CF_1 during illumination were described by several groups. For example, medium nucleotides accelerate the release of tightly bound ADP from membrane-bound ATP synthases. Starting with membranes containing [^{14}C]ADP on the tight binding site, Strotmann [20] found that ADP release at the onset of illumination is as fast as the formation of ATP; binding of medium ADP and phosphate to a site different from the tight site is a prerequisite for the rapid phase of nucleotide release.

Unfortunately, the rate of nucleotide exchange cannot be determined under the experimental conditions of Fig. 3, mainly because the release of AdN from site A occurs even after deenergization. Therefore it can not be excluded that site A gains full catalytic properties equivalent to site B after binding of substrates and ejection of bound ADP from site A; this would fit to the model of alternating sites involved in ATP synthesis ('energy-linked binding change mechanism') as developed by Boyer and co-workers [24,25]. In this model, ATP is formed from tightly bound ADP and phosphate without further energy input; binding of substrates to another site and an energy-depen-

dent conformational change leads to tight binding of ADP and phosphate, while the tight ATP site becomes a loose ATP site. After dissociation of ATP and another conformational change, the former tight site is able to bind substrate ADP and phosphate [24,25]. In this model, the different states of a binding site have different affinities for nucleotides; differences in relative affinities as described for site A and site B do not exclude the posibility that these sites are just different states of one and the same site. It is well accepted for the mitochondrial coupling factor that binding of ATP to one of three catalytic sites occurs with a very high affinity but decreases the affinity of the second binding site for ATP, thus showing negative cooperativity [39].

Exchange data found for phosphorylation are in agreement with a binding change mechanism (Refs. 20, 23, 24, and this paper) while the data on ATP hydrolysis/exchange (Fig. 6) are not. Further work is needed to find an explanation for the discrepancies between the data for ATP synthesis and for ATP hydrolysis.

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